
Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing *Enterococcus faecalis* within root canals and dentinal tubules

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Abstract

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Aim To evaluate the efficacy of 0.5%, 2.5% and 5.25% sodium hypochlorite (NaOCl) as intracanal irrigants associated with hand and rotary instrumentation techniques against *Enterococcus faecalis* within root canals and dentinal tubules.

Methodology A total of 180 extracted human premolar teeth were infected for 21 days with *E. faecalis*. The specimens were divided into 12 groups, as follows: group 1: 5.25% NaOCl + Hybrid technique (Valdrighi *et al.* 1998); group 2: 5.25% NaOCl + nickel–titanium (NiTi) rotary technique 4 mm shorter than the apex (by FOP-UNICAMP); group 3: 5, 25% NaOCl + NiTi rotary technique (Hero 642); group 4: 2.5% NaOCl + Hybrid technique; group 5: 2.5% NaOCl + NiTi rotary technique 4 mm shorter than the apex; group 6: 2.5% NaOCl + NiTi rotary technique (Hero 642); group 7: 0.5% NaOCl + Hybrid technique; group 8: 0.5% NaOCl + NiTi rotary technique 4 mm shorter than the apex; group 9: 0.5% NaOCl + NiTi rotary technique (Hero 642); group 10: sterile saline solution + Hybrid

technique; group 11: sterile saline solution + NiTi rotary technique 4 mm shorter than the apex; group 12: sterile saline solution + NiTi rotary technique (Hero 642). Canals were sampled before and after preparation. After serial dilution, samples were plated onto brain heart infusion (BHI) agar, and the colony forming units (CFU) that were grown were counted. The teeth were sectioned into three thirds and dentine chips were removed from the canals with conical burs. The samples obtained with each bur were immediately collected into test tubes containing BHI broth, and were incubated at 37 °C and plated onto BHI agar. The CFU were counted and analysed.

Results At all depths and thirds of the root canals and for all techniques used, 5.25% NaOCl was shown to be the most effective irrigant solution tested when dentinal tubules were analysed, followed by 2.5% NaOCl. No differences among concentrations in cleaning the canals were found.

Conclusions Especially at higher concentrations, NaOCl, was able to disinfect the dentinal tubules, independent of the canal preparation technique used.

Keywords: bacteria, dentinal tubules, instrumentation techniques, intracanal irrigants, sodium hypochlorite, root canal treatment.

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Introduction

Bacteria has long been recognized as the primary aetiological agent in the development of periapical bone lesions (Kakehashi *et al.* 1965). After root canal treatment, root canal failure from persistent infection

is a possibility (Sundqvist *et al.* 1998, Pinheiro *et al.* 2003). Reinfection or continued periapical inflammation of root-filled teeth may occur from viable bacteria residing within the root canal system and dentinal tubules (Safavi *et al.* 1990, Buck *et al.* 2001).

It has been demonstrated that eradication of infection enhances the success rate of the root canal treatment (Sjögren *et al.* 1997). The number of microorganisms within an infected root canal system may vary anywhere from 10^2 to $>10^8$ (Sjögren *et al.* 1991). Microorganisms are presents in all parts of the root canal system, including fins, anastomoses and may be found at varying depths of up to 300 µm within the dentinal tubules, from the pulpal end (Horiba *et al.* 1990). Berutti *et al.* (1997) histologically found bacteria deep in dentinal tubules after irrigation. Weiger *et al.* (2002) demonstrated *in vitro* that an infection time of 4 weeks allowed bacteria to penetrate human root dentine up to a depth of at least 150 µm. There also appears to be a regional variation in the extent to which dentine is invaded; cervical tubules are invaded to a greater extent than the midroot tubules, which are, in turn, invaded more than those in the apical region (Love 1996).

Enterococcus faecalis is the most commonly isolated species from the canals of teeth presenting post-treatment disease (Peciulienė *et al.* 2001). Pinheiro *et al.* (2003) found *E. faecalis* in 52.94% of canals with bacterial growth. This microorganism has demonstrated the capacity to survive in an environment in which there are scant available nutrients and in which commensality with other bacteria is minimal (Sundqvist *et al.* 1998).

Enterococcus faecalis can invade dentinal tubules (Ørstavik & Haapasalo 1990), and it is therefore probable that cells within dentinal tubules surviving chemo-mechanical instrumentation and intracanal medication could colonize the tubules and reinfect the obturated root canal (Kreft *et al.* 1992). Little is known about the mechanisms involved in bacterial invasion of dentinal tubules. However, dentinal tubules contain an appreciable amount of unmineralized collagen, and it has been established that dentinal tubule invasion by oral streptococci is associated with cell adhesion to collagen and a collagen-induced morphological growth response (Love *et al.* 1997). Love (2001) demonstrated that oral bacteria involved in dental caries and endodontic disease are able to gain nourishment from tissue fluids. This may account for the presence of streptococci and enterococci cases with post-treatment disease (Sundqvist *et al.* 1998, Pinheiro

et al. 2003), and suggests that tissue fluid from the periodontal ligament and alveolar bone bathing the root of the tooth may provide nutrition to bacteria within radicular dentinal tubules or the filled root canal for them to survive.

Among the procedures involved in the control of endodontic infection, irrigation is an important agent in eliminating microorganisms from the root canal system. Intracanal cleaning and disinfecting procedures are highly dependent on the mechanical and chemical effects of the irrigants. Irrigant solutions in different concentrations with antimicrobial activity have been used during biomechanical instrumentation, particularly sodium hypochlorite (NaOCl). To date, NaOCl is the most commonly employed root canal irrigant, but no general agreement exists regarding its optimal concentration, which ranges from 0.5% to 5.25%. Its antimicrobial property is proportional to the drug concentration (Gomes *et al.* 2001, Vianna *et al.* 2004), as well as its toxicity. The bactericidal ability of NaOCl results from the formation of hypochlorous acid (HOCl), when in contact with organic debris. HOCl exerts its effects by oxidizing sulphhydryl groups within bacterial enzyme systems, thereby disrupting the metabolism of the microorganism (Siqueira *et al.* 1997), resulting in the killing of the bacterial cells (Baumgartner & Cuenin 1992).

In the last 11 years, several new automated instrumentation system based on rotary nickel–titanium (NiTi) instruments have been proposed for root canal preparation. Rotary NiTi instruments with increased tapers and different designs have been recently developed. Moreover, most recently it has been observed that the different rotary NiTi instruments have shown inconsistent results with respect to cleaning ability (Hülsmann & Hahn 2000). Although it has been demonstrated that these newer instruments and techniques improve the shaping of the root canal, few studies have evaluated their ability in reducing the microorganisms within the canal root and dentinal tubules.

The purpose of this study was to evaluate the efficacy of 0.5%, 2.5% and 5.25% sodium hypochlorite as intracanal irrigants associated with three instrumentation techniques against *E. faecalis* within root canals and dentinal tubules.

Materials and methods

The method followed was a modification of one described previously (Gomes *et al.* 2003).

Selection and standardization of specimen

A total of 120 extracted human mandibular premolar teeth with a single root canal, checked by radiographs, were selected for this experiment. Their crowns were removed with a water-cooled diamond saw and their roots were standardized to a length of 15 mm. The external surface of all teeth and the cementum were cleaned with periodontal curettes. The root canals and the apical foramen were enlarged with K-files up to size 20, under irrigation with tap water. The smear layer formed in the canal walls during the instrumentation was removed using an ultrasonic bath with 17% EDTA for 10 min followed by 5.25% NaOCl for 10 min and tap water for 1 h as outlined by several authors (Ferraz *et al.* 2001). The efficiency of the method was observed in a pilot study by SEM where it was possible to verify the presence of open dentinal tubules.

Specimen sterilization

The teeth, in groups of 5, were placed into glass tubes containing 5 mL of brain heart infusion (BHI, Oxoid, Basingstoke, UK) broth medium and autoclaved at 121 °C, 1 atm, for 20 min. Then, they were kept in an incubator at 37 °C for 48 h to check the efficacy of the sterilization treatment.

Cultivation of the *Enterococcus faecalis* and specimen contamination

Pure cultures of *E. faecalis* (ATCC 29212) were previously cultivated in BHI broth for 24 h, and then cultured in 5% defibrinated sheep blood + BHI agar plates. Suspensions of this bacterium had the optical density adjusted spectrophotometrically at 800 nm (Optical D_{800}) to match the turbidity of 6×10^8 CFU mL⁻¹ (equivalent to 2.0 McFarland standard). The glass tubes were opened inside a laminar flux chamber. Sterile pipettes were used to remove 5 mL of sterile BHI and to replace it with 5 mL of the bacterial inoculum. The flasks were closed and kept at 37 °C for 21 days (Fuss *et al.* 2002). The medium was changed every 2 days to avoid saturation and confirm the growth of *E. faecalis*. The turbidity of the medium during the incubation indicated bacterial growth (Fig. 1). The purity of the cultures was confirmed by Gram staining, catalase production, colony morphology on BHI agar + blood and by the use of a biochemical identification kit (API 20 Strep, BioMérieux SA, Marcy-L'Etoile, France) after 21 days.

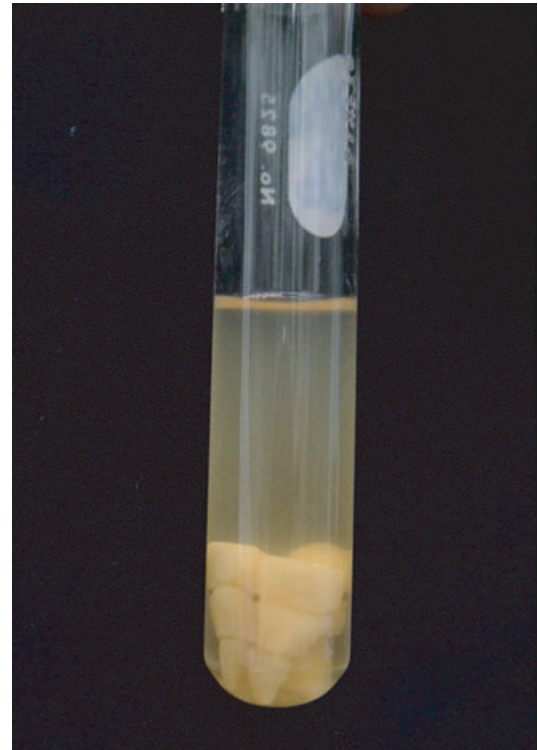


Figure 1 Sample contamination with *Enterococcus faecalis*.

If any contaminants would have been discovered, the teeth would be rejected. Bacterial penetration into the dentinal tubules using this technique was confirmed by SEM in a pilot study.

Experimental groups

After contamination, the specimens were mounted in a sterile aluminium apparatus and then randomly divided into 12 groups, according to the intracanal irrigant and instrumentation technique used, as follows:

- Group 1: 5.25% NaOCl + Hybrid technique.
- Group 2: 5.25% NaOCl + FOP-UNICAMP technique.
- Group 3: 5.25% NaOCl + NiTi rotary technique Hero 642 (MicroMega, Besançon, France).
- Group 4: 2.5% NaOCl + Hybrid technique.
- Group 5: 2.5% NaOCl + FOP-UNICAMP technique.
- Group 6: 2.5% NaOCl + NiTi rotary technique Hero 642 (MicroMega).
- Group 7: 0.5% NaOCl + Hybrid technique.
- Group 8: 0.5% NaOCl + FOP-UNICAMP technique.
- Group 9: 0.5% NaOCl + NiTi rotary technique Hero 642 (MicroMega).

- Group 10: sterile saline solution + Hybrid technique.
- Group 11: sterile saline solution + FOP-UNICAMP technique.
- Group 12: sterile saline solution + NiTi rotary technique Hero 642 (MicroMega). Groups 10, 11, 12 were the positive control groups.

The teeth in groups 1, 4, 7 and 10 were instrumented using the Hybrid technique as described by Valdrighi *et al.* (1998). The cervical two-thirds (10 mm) of the canal were prepared initially using K-files (DYNA-FFDM endodontic instruments, Bourges, France) up to size 35. A Gates Glidden (GG) bur (DYNA-FFDM endodontic instruments) size 2 was then used with gentle force up to this length, followed by a GG size 3 one mm shorter (7 mm). A K-file size 10 (DYNA-FFDM endodontic instruments) was used to recapitulate the canal 1 mm beyond this length between each file and bur in order to maintain patency. Apical instrumentation was commenced with a straight file of the same size as the apical foramen (K-file 20). The instrument was used with a half turn reaming action until the file became loose within the canal. A size 35 was used to establish the apical stop for the teeth (14 mm). Stepback flaring of the canal was performed using larger files at intervals manipulated in a filing action. The file used to prepare the apical stop was used to recapitulate. Stepping back ended after the use of 3 files larger (K-file 50) than the file that prepares the apical stop.

The teeth in groups 2, 5, 8 and 11 were instrumented using the crown-down principles, combining Gates Glidden burs with NiTi Hero (Taper 0.06, tip 20) instruments at a constant speed of 350 rpm. Initially, the cervical two-thirds (10 mm) of the canal were prepared using a Hero instrument. Then, Gates Glidden burs sizes 5–2 were used. Each one was introduced 2 mm within the root canal beyond the preparation of the preceding bur. The apical stop was established using files up to a size 35. Step-back flaring of the canal was performed using larger files at intervals manipulated in a filing action. The file used to prepare the apical stop was used to recapitulate. Stepping back ended after the use of 3 files larger (K-file 50) than the file that prepared the apical stop.

The teeth in groups 3, 6, 9 and 12 were prepared according to the protocol described by the manufacturer of the Hero system (MicroMega) for straight canals, as follows: at first, the cervical two thirds were prepared with Hero taper size 0.06 (10 mm), tip size 30. Then, the Hero taper 0.04, tip 30, was used to instrument 2 mm shorter than the apical foramen

(13 mm). After these procedures, the Hero taper 0.02, tip 30, was introduced into the canal to the full length of the canal (15 mm). The Hero instruments were set into permanent rotation (350 rpm) with a 16:1 reduction handpiece powered by an electric motor (Maillefer Dentsply, Ballaigues, Switzerland).

Irrigation

For the Hybrid and FOP-UNICAMP techniques, the following irrigation regimens were used: Prior to a new instrument, the canal was irrigated with 0.5 mL of the auxiliary chemical substance (0.5%, 2.5% or 5.25% NaOCl). After the use of each instrument, 1.0 mL of saline solution was used as irrigating solution.

For the Hero 642 technique, the followed irrigation regimens were used: prior to a new instrument, the canal was irrigated with 2.0 mL of the auxiliary chemical substance (0.5%, 2.5% or 5.25% NaOCl). After the use of each instrument, 4.0 mL of saline solution was used as irrigating solution.

In relation to the group control (saline), in the Hybrid and FOP-UNICAMP techniques, the same amount of saline (0.5 mL) was used before each new instrument and 1.0 mL after the use of each instrument. Regarding the Hero technique, 2 mL of saline were used before each new instrument and 4.0 mL after the use of each instrument, so that all techniques would have the same final volume. The final volume of the irrigant solutions (NaOCl and saline) was 18 mL in each one of the three techniques tested. The needle (28-gauge) was placed up to the apical third without binding.

Microbial sampling of the canals

Canals were sampled before and after instrumentation using three sterile paper points. After chemo-mechanical preparation, irrigation with 5% sodium thiosulphate solution was used to neutralize the NaOCl. Subsequent to each sampling, the paper points were transferred to tubes containing 1 mL of BHI broth and vortexed for 1 min. After 10-fold serial dilutions, aliquots of 0.1 mL were plated onto BHI + blood agar plates and incubated at 37 °C for 48 h. The colony forming units (CFU) grown were counted.

Dentinal samples

Besides this, each tooth was sectioned in three thirds and dentine chips were removed from the canals using

increasing-diameter sterile diamond-tipped conical burs [3069 (ISO 018), 3139 (ISO 021), 4137 (ISO 025), 720 G (ISO 029), KGSorensen, São Paulo, SP, Brazil] driven by an electric motor at low speed (Maillefer Dentsply). The last bur (720 G) was used only in the cervical third (the largest third). The samples obtained with each bur and in each third were immediately collected into separate test tubes containing BHI broth. In order to standardize the volume of the dentine chips removed by each bur, the BHI flasks were weighed before and after dentine collection. Then, the tubes were incubated at 37 °C for 48 h and plated onto BHI agar. All assays were repeated three times. The CFU were counted and the purity of the positive cultures was confirmed by Gram staining, catalase production, colony morphology on BHI blood agar and by using a biomechanical identification kit (API 20Strep, Bio-Mériux SA, Marcy-l'Étoile, France).

Table 1 Reduction of average CFU mL⁻¹ from the first to second microbiological sampling (before and after instrumentation techniques) of *Enterococcus faecalis* within root canals

	Hybrid (%)	FOP-UNICAMP (%)	Hero 642 (%)
5.25% NaOCl	100	100	100
2.5% NaOCl	100	100	99.99
0.5% NaOCl	99.99	99.98	99.98
Saline solution	99.56	99.92	99.32
Total	99.88	99.97	99.82

Statistical analysis

The data, which were nonparametric, were statistically analysed using the Kruskal–Wallis test and Dunn test (*post hoc*) (Biostat Program, CNPq, 2000, Brasília, DF, Brazil). The level of significance was set at 0.05.

Results

Table 1 shows the average percentage reduction of *E. faecalis* in CFU mL⁻¹ inside the root canal after the biomechanical procedures tested. There were no significant differences between any of the tested groups ($P > 0.05$).

In Table 2, the results were displayed according to the thirds and at all depths. In all thirds and for all techniques, 5.25% NaOCl was shown to be the most effective irrigant solution tested, followed by 2.5% NaOCl. These concentrations had statistically significant differences between themselves only at depth 3 (all thirds and techniques) and in the apical and cervical thirds of depth 2 using Hero 642.

There were no statistically significant differences in the weight of dentine collected at each of the depths (1, 2, 3 and 4) produced by burs 3069, 3139, 4137, 720 G respectively, indicating that each bur, independent of its size, removed the same amount of dentine.

The results in Table 3 are related to the average CFU mL⁻¹ counted after treatment with irrigant solutions at all depths of dentine, in all thirds and for the

Table 2 Median of CFU mL⁻¹ of *Enterococcus faecalis* in dentin samples divided into depths, thirds and techniques

Portion of tooth and solutions	Depths and techniques								
	1			2			3		
	Hybrid	FOP-UNICAMP	Hero 642	Hybrid	FOP-UNICAMP	Hero 642	Hybrid	FOP-UNICAMP	Hero 642
Cervical									
5.25% NaOCl	0d	0d	0c	0e	0c	0d	0f	0d	0e
2.5% NaOCl	0d	0d	0c	0e	0c	0c	40cde	0bc	0d
0.5% NaOCl	40bc	0c	0b	40b	40b	40a	40b	40ab	80a
Saline solution	320a	40a	400a	280a	80a	80a	360a	80a	80a
Medium									
5.25% NaOCl	0d	0d	0c	0e	0c	0d	0f	0d	0e
2.5% NaOCl	0d	0d	0c	0d	0c	0d	40de	40bc	0cd
0.5% NaOCl	40bc	0bc	0b	40bc	40b	40b	40bcd	40bc	0cd
Saline solution	80b	40a	40a	40b	80a	120a	80b	80bc	80ab
Apical									
5.25% NaOCl	0d	0d	0c	0e	0c	0d	0f	0d	0e
2.5% NaOCl	0d	0d	0c	0e	0c	0c	0e	0b	0d
0.5% NaOCl	40c	0c	0b	40cd	40b	40b	40e	40c	40c
Saline solution	80b	40ab	40a	80b	40b	40b	40bc	0c	40b

Different letters (from a to f) indicate significant difference in vertical direction (Kruskal–Wallis $P < 0.05$).

Table 3 Median of CFU mL⁻¹ of *Enterococcus faecalis* in all thirds

Solution	Depths and techniques											
	1			2			3			4		
	Hybrid	FOP- UNICAMP	Hero 642	Hybrid	FOP- UNICAMP	Hero 642	Hybrid	FOP- UNICAMP	Hero 642	Hybrid	FOP- UNICAMP	Hero 642
5.25% NaOCl	0 cA	0cA	0cA	0dA	0dA	0dA	0dA	0dA	0dA	0 bA	0 bA	0 cA
2.5% NaOCl	0 cA	0cA	0cA	0cA	0cA	0cA	40cA	0cA	0cA	40 aA	40 aA	0 bA
0.5% NaOCl	40 bA	0bA	0bA	40bA	40bA	40bA	40bA	40acA	40bA	40 aA	40 aA	40 aA
Saline solution	120aA	40aA	40aA	120aA	40aA	40aA	80aA	40aA	40aA	160 aA	40 aA	40 aA

Different letters (from a to d and A) indicate significant difference (Kruskal–Wallis $P < 0.05$). Capital letters indicate differences in horizontal direction. Lower case letters indicate differences in vertical direction.

three instrumentation techniques tested. Significant differences were seen between the concentrations of NaOCl and the positive control group (saline solution). For the last depth (depth 4), presented only in the cervical third, the groups where 0.5% NaOCl was tested did not present significant differences when compared with the positive control groups. The instrumentation techniques tested in this study had the same performance ($P > 0.05$).

Discussion

There are several reported methods for evaluating endodontic instrumentation techniques. A bacteriological assessment was selected because of the importance of canal disinfection in successfully treating apical periodontitis. Although mechanical instrumentation reduced bacteria from human root canals by approximately 50%, auxiliary substances may be necessary to aid the removal of the microbiota in areas where instruments cannot reach (Lee *et al.* 1990).

Microorganisms can invade the dentinal tubules of both teeth with vital and nonvital pulps, but the invasion is less severe with vital pulps because of the protective function of the pulp (Nagaoka *et al.* 1995). Bacteria entering either the pulpal surface or periodontal surface of the root can exist within the dentinal tubules. If viable, these bacteria could act as reservoirs of infection (Buck *et al.* 1999).

Enterococcus faecalis, a facultatively anaerobic Gram positive coccus, was chosen as a test organism as it is associated with persistent apical inflammation and is often found existing in monocultures (Pinheiro *et al.* 2003). This microorganism seems to be the best organism to perpetrate experimental penetration into dentinal tubules, leading to gross infection (Peters *et al.* 2000, Dametto *et al.* 2005). It has been used in previous studies on the efficacy of irrigant solutions and intracanal medications (Gomes *et al.* 2003) espe-

cially for its high level of resistance to a wide range of antimicrobial agents (Gomes *et al.* 2001).

Some methodologies permit direct contact between the antimicrobial substances and the microorganisms (Gomes *et al.* 2001, Vianna *et al.* 2004) but in this study, and those of Dametto *et al.* 2005 and Menezes *et al.* 2004, who tested intracanal medications, the microorganisms were located within the dentinal tubules and the antimicrobial agents did not necessarily have direct contact with the microorganisms.

NaOCl solution has been used for >70 years because of its well known antimicrobial action and its ability to dissolve tissue, but no general agreement exists regarding its optimal concentration, which ranges from 0.5% to 5.25%. Clinical and laboratory studies have not demonstrated any significant difference in antibacterial effect between NaOCl concentrations ranging from 0.5% to 5% (Siqueira *et al.* 2000) in the root canal (canal wall samples). However, in the present study, when the dentinal tubules were evaluated, there were statistically significant differences between the concentrations tested.

With the reduced working time made possible by the advent of rotary instruments and techniques for root canal preparation, the irrigant of choice should be one that exerts its microbial activity quickly against resistant microorganisms found in the root canal and dentinal tubules, such as *E. faecalis*. In this study, all concentrations of NaOCl tested significantly reduced the microorganisms within dentinal tubules and in the root canal in a period of 10 min.

Studies have shown that debris and bacterial content is reduced in almost 95% of the cases (Dametto *et al.* 2005) by the flushing action of saline solution. However, its effectiveness is directly related to the volume and frequency of irrigation as well as to the depth of the irrigating needle. These facts were confirmed by the present study, where 98% bacteria reduction was found using a higher volume of saline associated with the instrumentation.

Irrigants and irrigation are extremely important for debridement of the canal space. To be most effective, several aspects must be considered: flushing, chemical and antimicrobial action, type of solution and delivery systems (Walton 1992). Usually the flushing action, which is the physical removal of debris from the canal space, should be performed by sterile saline solution, which is much more similar to body fluids than distilled water or other chemical agents. Studies have shown that debris and bacterial content is reduced in almost 95% of the cases (Dametto *et al.* 2005) by the flushing action with saline solution. However, its effectiveness is directly related to the volume and frequency of irrigation as well as the depth of the irrigating needle.

In the present study, the microbiological samples collected within the root canals with paper points were obtained just after biomechanical preparation in order to evaluate the chemico-mechanical action immediately after the instrumentation. Similarly, the dentinal samples were obtained using burs of different diameters to evaluate the presence of bacterial cells inside the dentinal tubules just after the biomechanical procedures. The results showed that instrumentation techniques and irrigation with saline solution, without any antimicrobial action, removed >95% of the bacterial cells from the root canal, agreeing with the findings of Siqueira *et al.* (1999).

Many studies have evaluated the contact time required by irrigant solutions to produce negative cultures (Gomes *et al.* 2001, Radcliffe *et al.* 2004, Vianna *et al.* 2004). Others observed inhibition zones formed on agar plates containing different bacterial species (Conti *et al.* 1999). Some authors verified the bacterial reduction produced by different techniques and/or different irrigating solutions in the root canal (Siqueira *et al.* 1999). But none of them evaluated bacterial reduction inside dentinal tubules immediately following chemo-mechanical preparation.

The present study showed that the absence of bacterial growth in the samples immediately after biomechanical preparation (main canal samples) did not deflect on absence of bacterial growth in the dentinal samples (dentinal-tubule samples), even after the use of irrigants with antimicrobial action, such as 0.5% and 2.5% NaOCl.

Conclusion

The results of the present study suggest that 5.25% NaOCl has a greater antibacterial activity inside the

dentinal tubules infected with *E. faecalis* than the other concentrations tested. Under the conditions of this study, irrigants such as NaOCl seem to be able to penetrate well into dentinal tubules.

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